

Original Article

Study of specific IgE and IgG₄ antibodies to mite antigen in asthmatic children during immunotherapy

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ABSTRACT

Using immunoblotting and densitometer techniques, we followed the levels of IgE and IgG₄ antibodies to *Dermatophagoides farinae* antigen in the sera of 12 asthmatic children during immunotherapy, repeated injections of house dust extract, for 2 years. The symptoms of asthma were reduced or disappeared by the 6th month after starting immunotherapy in 12 children. Two years later, RAST scores to house dust and *D. farinae* in each child were still 4 or 5. The levels of IgE antibodies to *Der fII* in all cases did not change drastically. IgE antibodies to higher molecular weight antigens (*Der fI*, *fIII*, *fIV* etc.) developed in some cases, suggesting that repeated injections of crude extract tended to induce IgE antibodies to alternative molecules in patients. The IgG₄ antibody to *Der fII* increased in 10 cases, while that to *Der fI* increased in three of 12 cases, indicating that production of IgG₄ antibodies to mite antigens started and expanded with repeated injections of house dust extract. The amount of induced IgG₄ antibodies to *Der fI* and *Der fII* was not enough to inhibit the immunoblot reaction of IgE antibodies to these molecules.

Key words: bronchial asthma, densitometer, *Dermatophagoides*, IgE, IgG₄, immunoblotting, immunotherapy.

INTRODUCTION

Allergen immunotherapy, repeated injections of allergen to patients, has been practised for more than 80 years.¹ The efficacy of immunotherapy has been reported by double blind tests,^{2–5} although its mechanism has not been clarified as yet. A possible role has been suggested in which repeated injections of allergen induce blocking antibodies that compete for epitope sites on the allergen molecule with IgE antibodies on the mast cell surface.^{6,7} During house dust extract immunotherapy for patients with nasal allergies or bronchial asthma due to house dust mites, levels of IgE and IgG antibodies have been measured by radio-allergosorbent test (RAST) and enzyme-linked immunosorbent assay (ELISA) using crude mite extract as an antigen. In most patients, IgE antibody levels to crude mite extract do not change, although IgG antibody levels, in particular IgG₄, increase during immunotherapy. Several reports have indicated that the IgG₄ subclass works as a blocking antibody.^{8–12}

If sufficient IgG antibody to the mite allergen molecule is induced, IgE antibody levels to the molecule should be reduced, because competition for the epitope sites on the molecule will occur between IgE and IgG antibodies. There are two possible reasons to explain why IgE levels to crude mite antigen in *in vitro* tests do not change during immunotherapy. One is that the levels of blocking antibody are not high enough to inhibit the reaction between IgE antibody and the antigen under *in vitro* conditions. The other is that IgE antibodies to alternative molecules of mite antigen are induced by repeated injections of crude house dust extract.

Mite antigen consists of numerous molecules inducing IgE and IgG^{13,14} and not all people may react to these

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molecules in the same manner. It is necessary to follow IgE and IgG antibody levels to each molecule of mite antigen during immunotherapy. Immunoblot analysis demonstrates numerous allergens in mite extract and helps us to recognize the polymorphism of humans in the recognition of mite antigen. Of these allergens, *Der fl* and *Der pl* (24 kDa) as well as *Der fII* and *Der pII* (15 kDa) are considered major allergens.^{15,16} More than 50% of atopic sera to mite extract react with these major allergens. In the present report we used immunoblotting techniques to follow levels of specific IgE and IgG₄ antibodies in the sera of allergic patients during immunotherapy.

METHODS

Patients and immunotherapy

Twelve children, who consulted a pediatric clinic periodically for treatment of bronchial asthma and had not received immunotherapy previously, were chosen to participate in the study. At the beginning of immunotherapy, their ages were ranged from 5 to 12 years (mean 7.5 years). RAST scores to *Dermatophagoides* species were 4 or 5. Sera were taken on the first day of injection and at 6, 12, 18 and 24 months after starting immunotherapy.

House dust extract for injection was dispatched by Torii Co. (Tokyo, Japan). The first dose of injection was 0.1 mL at a dilution of 10^{-6} . Injections were repeated once a week and the dose of house dust extract was increased; for example, 0.1, 0.2, 0.4, 0.7 and 1.0 mL at a dilution of 10^{-6} , 0.1, 0.2 ... 1.0 mL at a dilution of 10^{-5} and so on. By the sixth month, the amount of injection reached 0.4 mL at a dilution of 10^{-1} . This final dose was injected every other week as a maintenance dose. If the skin reaction was too severe at this dose, the amount or concentration of the extract for injection was reduced.

Preparation of mite extract for immunoblot analyses

Dermatophagoides farinae was cultured according to the method of Sasa *et al.*¹⁷ and the extract was prepared according to previously described methods.¹⁸ Briefly, 1 g cultured medium containing approximately 20 000 mites was mixed in 10 mL of 0.02 mol/L Tris-HCl buffer (pH 7.6), homogenized with a Teflon-glass homogenizer and centrifuged at 3000 g for 20 min. The supernatant was used as a crude mite extract. Protein concentration was determined with a protein assay kit

(Protein Assay Kit I; Bio-Rad, Hercules, CA, USA) using bovine serum globulin as a standard.

Immunoblotting

Crude mite extract (approximately 500 µg protein) was applied to the top of a stacking gel, horizontally prepared without combs and separated on a gradient polyacrylamide gel (5–15%, 16 × 16 cm) under non-reducing and non-boiling conditions.¹⁹ Separated proteins were transferred to a nitrocellulose sheet for immunoblotting. To confirm the proper separation and transfer of proteins, both edges of a 10 mm nitrocellulose sheet was cut and stained with Coomassie Brilliant Blue. The remaining central part of the nitrocellulose sheet was immersed in 5% skimmed milk in phosphate buffered saline (SM/PBS) and cut into 20 strips at a width of 7 mm. Two strips were put in a plastic bag and 2 mL patient's serum diluted 1:5 with SM/PBS containing 0.05% Tween 20 (SM/PBS/Tw) was added. For one patient, five plastic bags were prepared to incubate nitrocellulose strips and the sera at 0, 6, 12, 18 and 24 months under the same conditions. The bags were sealed and incubated overnight at 4°C. The next day, strips were washed with PBS/Tw five times. One of the two strips in each bag was incubated with [¹²⁵I]-labeled anti-human IgE (Pharmacia RAST Kit; Uppsala, Sweden) and the other strip was incubated with anti-human IgG₄ conjugated to horseradish peroxidase (Tago, Camarillo, CA, USA; 1:1000 diluted with SM/PBS/Tw). Three hours later, strips were washed with PBS/Tw four times. Strips were dried and exposed to X-ray film (Fuji Co., Tokyo, Japan) for 7–14 days at –80°C to detect IgE. Strips were reacted with a substrate kit (Konica Immuno Stain HRP-1000; Konica Co., Tokyo, Japan) to detect IgG₄.

Density scanning

The density of *Der fl* and *Der fII* bands on X-ray films or nitrocellulose sheets was scanned by a densitometer (Shimazu CS-9000; Shimazu Co., Kyoto, Japan) and changes in antibody levels were traced. To test the correlation between serum dilution and the density of the bands, one patient's serum was variously diluted (1:2.5, 1:5, 1:10 and 1:20) and incubated with nitrocellulose strips, on which mite antigens were separated. Immunoblotting was performed with anti-human IgG₄ as mentioned earlier and the density of the bands was scanned.

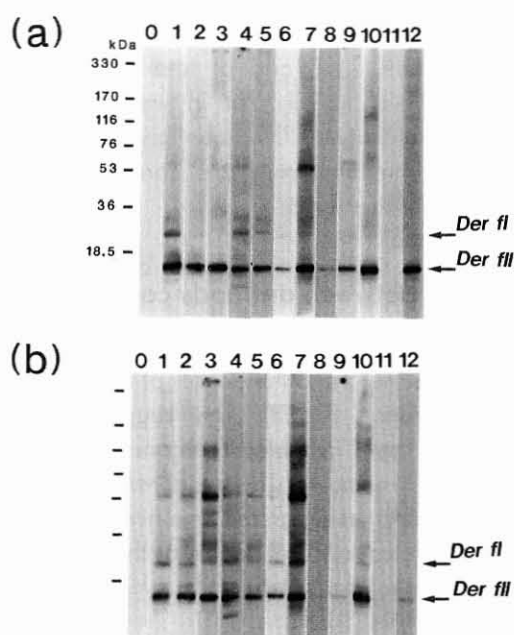


Fig. 1 Autoradiography of mite antigen reacted with IgE antibody in individual serum (a) before starting and (b) after 24 months of immunotherapy. Lane 0, non-atopic serum; lanes 1–12, sera of asthmatic children

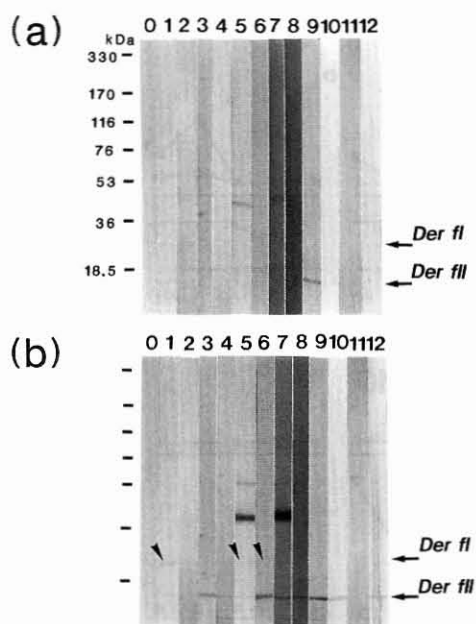


Fig. 2 Nitrocellulose sheets with bands of mite antigens reacted with IgG₄ antibody in individual serum (a) before starting and (b) after 24 months of immunotherapy. Lane 0, non-atopic serum; lanes 1–12, sera of asthmatic children. IgG₄ antibodies to *Der fl* increased in three of 12 cases (nos 1, 5 and 6; arrows) and those to *Der fll* increased in 10 of 12 cases (nos 1, 3–10 and 12).

RESULTS

Symptoms of bronchial asthma

Symptoms of bronchial asthma in 12 children were reduced or disappeared by the sixth month after starting immunotherapy and few symptoms appeared until the 24th month. It was correlated to the development of IgG₄ to *Der fll*. Because we did not prepare control groups, such as a group of placebo-injected patients or a group of patients receiving medication only, we do not know how much immunotherapy contributed to the reduction of asthmatic symptoms. The RAST scores to house dust and *D. farinae* in the sera of 12 children were maintained at 4 or 5 until the 24th month.

Reactivity of mite antigen to IgE and IgG₄ antibodies in patients' sera

Many bands appeared on X-ray film when separated mite antigen was reacted with patients' sera and probed with [¹²⁵I]-labeled anti-human IgE (Fig. 1a). Patterns of bands were different in each patient. Among many bands, the 15 kDa band (*Der fll*) and the 24 kDa band (*Der fl*)

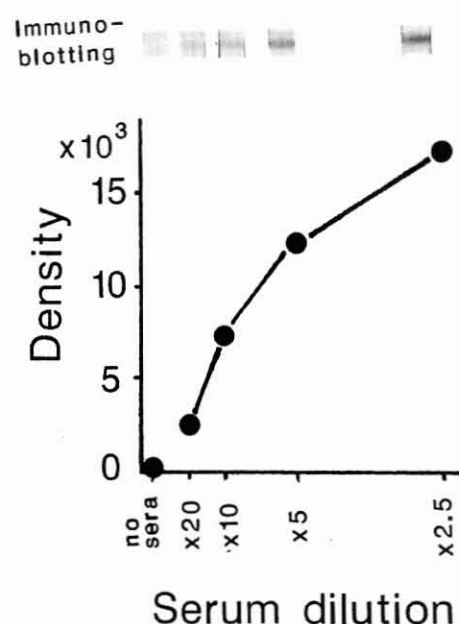


Fig. 3 Correlation between serum concentration and density of the band of *Der fll* reacting to IgG₄ antibody. Serum of patient no. 9 at the 24th month was diluted ($\times 2.5$, $\times 5$, $\times 10$, $\times 20$) and reacted with nitrocellulose sheets on which mite antigen was separated. Density of *Der fll* band was measured.

appeared frequently. In 12 patients, 11 cases reacted with *Der fl* and nine cases reacted with *Der fl*. When pre-immunotherapy sera and sera from the 24th month after immunotherapy (Fig. 1a,b) were compared, the densities of the 15 kDa (*Der fl*) bands did not differ in each patient. However, IgE antibodies to higher molecular weight antigens (e.g. 24, 29, 56, 120 and 170 kDa) developed in several patients. Molecules of 24, 29 and 56 kDa may be major mite allergens of *Der fl*, *flII* and *flV*, respectively. Possibly, these IgE antibodies were induced by repeated injections of crude house dust extract. Symptoms of asthma in the patients who developed IgE antibodies to higher molecular weight antigens were controlled as well as in patients who did not develop IgE antibodies to these molecules.

Before starting immunotherapy, few bands reacted with IgG₄ antibodies (Fig. 2a). After repeated injections of

house dust extract, IgG₄ antibodies to *Der fl* increased in three cases (nos. 1, 5 and 6) and those to *Der flII* increased in 10 of 12 (Fig. 2b). In two patients (nos. 5 and 7), IgG₄ antibody to 40 kDa antigen developed after 6 months of immunotherapy and continued at similar levels until the 24th month.

Correlation between antibody concentration and band density

Series of diluted sera and the density of bands showed a positive correlation (Fig. 3). This suggested that the density of the band indicated the concentration of the antibody to the molecule. This experiment also demonstrated that molecules separated on a nitrocellulose sheet were not saturated with antibodies at the concentration used in the present study (1:5).

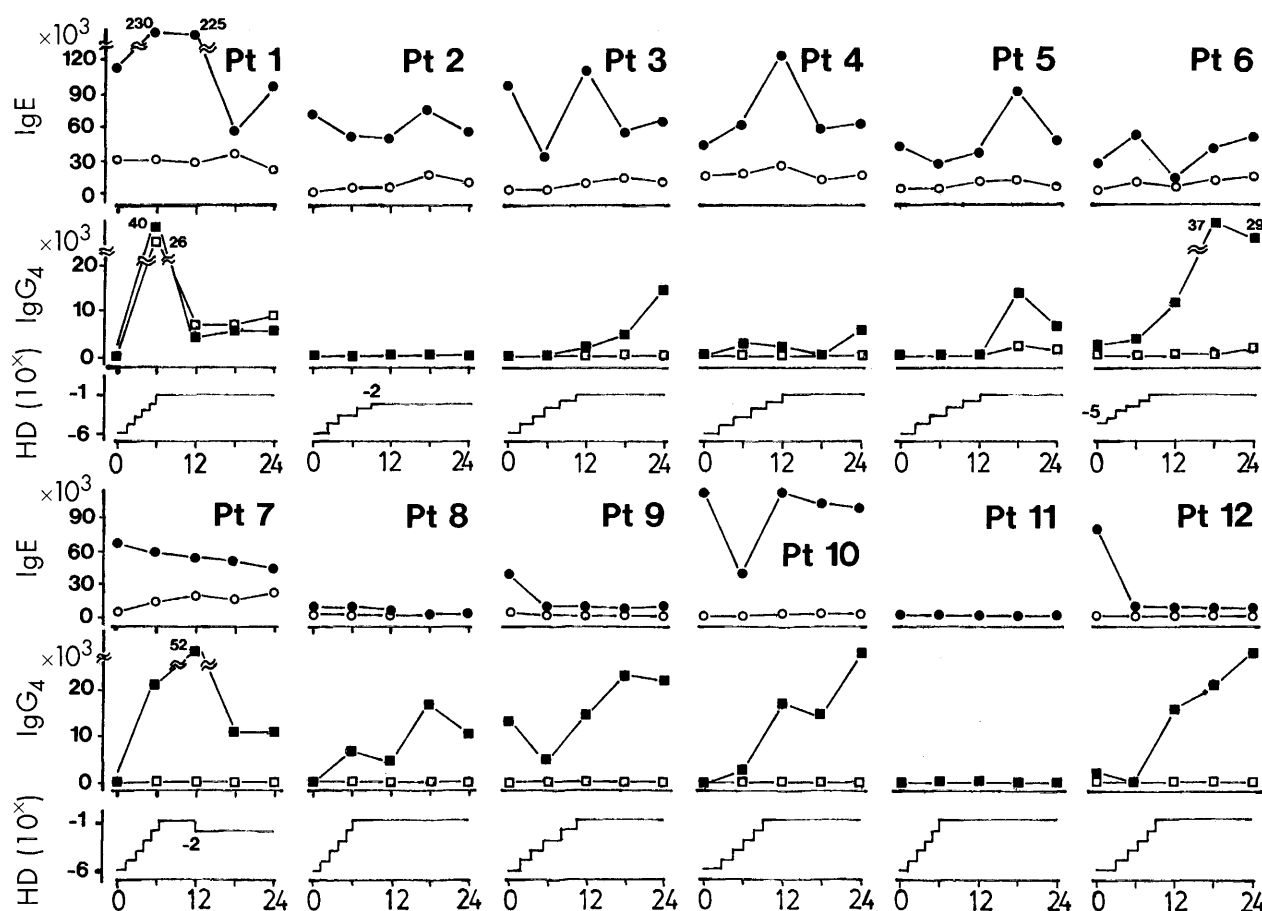


Fig. 4 Changes in the density of *Der fl* (open symbols) and *Der flII* (closed symbols) bands reacting with IgE (○, ●) or IgG₄ (□, ■) during immunotherapy in each patient (Pt). The dose of injection is individually drawn below. HD, house dust extract.

Changes of density in *Der fl* and *Der fII* bands reacted with IgE or IgG₄ antibodies during immunotherapy

In almost all patients, the density of *Der fII* bands to IgE antibodies changed slightly, but not significantly (Fig. 4). The density of *Der fII* bands was much higher than that of *Der fl* bands in all patients. Two patients (nos. 9 and 12) showed significant decreases in IgE to *Der fII* and increases in IgG₄ to the molecule, suggesting that competition may occur between IgE and IgG₄ on the *Der fII* molecule.

IgG₄ antibodies to *Der fl* and *Der fII* were detected at the sixth month. The levels increased and were stable from the 12th to the 24th month. This suggests that repeated injections stimulate the production of IgG₄ antibodies to *Der fl* and *Der fII*.

DISCUSSION

Results from previous double-blind studies using crude mite extract and placebos for injection have supported the efficacy of immunotherapy.²⁻⁵ Groups injected with mite extract showed a better clinical response than groups injected with placebo. Antibody levels of specific IgG to crude mite extract increased in the mite extract-injected group but not in the placebo group. Specific IgE levels to crude mite extract did not change in either group. Because those studies used crude mite extract for injection and evaluation, it was impossible to analyze which molecule in the crude mite extract reacted with IgE antibodies and which molecule in the extract induced IgG antibodies in the injected patients. Protein separation techniques resolved this problem. The western blotting technique indicates specific molecules of antigen that react with IgE and IgG antibodies.^{13,14} Little work has been done with western blotting to look for molecules in the mite extract that induce IgE and IgG antibodies during immunotherapy. We attempted to analyze changes in IgE and IgG₄ antibodies to the molecules, in particular, *Der fl* and *Der fII*.

Injection of house dust extract to asthmatic children did not affect the production of IgE antibodies to *Der fII* during the observation period. However, IgE antibodies to higher molecular weight antigens (e.g. 120 and 170 kDa) were induced in some cases, in whom the symptoms of asthma were still well controlled. We believe that repeated injections of house dust extract induced IgE antibodies to alternative molecules, in particular high

molecular weight antigens. These high molecular weight molecules are thought not to be able to move from airways to immune centers, such as lymph nodes, because of their size. Mucocutaneous barriers prevent these large molecules from moving out of the airways. However, if these molecules are injected, they may be immunogens that induce immunoglobulins, including IgE antibody. In Japan, we use house dust extract as an antigen for injection. It contains numerous antigens. Thus, patients are injected with many allergens, which may cause alternative allergic disorders. To avoid induction of alternative IgE antibodies, we would like to recommend that a purified antigen, rather than a crude antigen, is injected for immunotherapy.

Competition between IgE and IgG₄ to *Der fl* or *Der fII* seldom occurred in the analysis method we used in the present study. The amount of antigen on the nitrocellulose sheet may be enough to bind both IgE antibodies and competitive IgG₄ antibodies. This explains the RAST score during immunotherapy. Discs for RAST contain much antigen and competition between IgE and IgG₄ may not occur. This is why the RAST score does not change during immunotherapy, even though sufficient amounts of IgG₄ antibodies were induced to neutralize allergic reactions.

An extraordinary amount of IgG antibodies is needed to block antigen *in vivo* at the mucocutaneous lesion before attaching the antigen with IgE antibodies on the mast cell surface. Our experiments do not support this possibility. IgG levels induced by the injections were not sufficient to prevent mite allergens from binding to specific IgE antibodies on mast cells. However, mast cell degranulation can be inhibited if cross-linkage of IgE antibodies on the cell surface is inhibited by IgG antibodies. If IgE antibody on a mast cell captures one antigen molecule but a free IgG antibody attaches to the captured molecule on another site, another IgE antibody on the cell cannot attach the antigen molecule. As a result, cross-linkages are not formed and degranulation does not occur. This can occur when the amounts of IgG and IgE antibodies are similar, if the affinity of both antibodies to the antigen is identical. This likely explains the effect of immunotherapy according to our results; however, further study is needed to confirm this.

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